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Nanoparticle ecotoxicity – physical and/or chemical effects?

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The increased production and use of engineered nanoparticles (ENPs) within the last decade has caused concern about their potential adverse impacts on the aquatic environment. For the purpose of hazard identification, Organization for Economic Cooperation and Development (OECD) test guidelines and International Standards Organization (ISO) standard tests are used to identify and compare the aquatic toxicity of chemical substances. These test methods have been developed for soluble chemicals and since ENPs are suspended rather than dissolved in the test medium, the validity of the tests is challenged. ENPs in aqueous suspension undergo various time-dependent processes that potentially modify their availability and stability during the exposure period (Figure 1, A-G), thereby violating the common assumption of stable exposure over the duration of the test. This is one of the main reasons why standard tests may not be appropriate for ENPs, at least not without certain considerations and modifications to the test setup.

Furthermore, these processes may help explain why aquatic toxicity testing of ENPs generally is affected by poor reproducibility and altered concentration-response patterns compared to soluble compounds (Hartmann et al. 2013). For example, poor reproducibility related to time-dependent changes in exposure concentrations has been shown for silver nanoparticles (AgNPs) (Sørensen and Baun 2015). The toxicity, and indirectly the bioavailability, of AgNPs were tested in a 2h algal test, 0-5 days after preparation of the AgNP suspension. The toxicity was found to increase within the first 48h upon preparation of the suspensions, and decrease in the following 72h period, likely due to dissolution and aggregation.

ENPs may also interfere with the test endpoint by indirectly contributing to the measured effect via seemingly non-toxicological mechanisms. The endpoints recorded in guideline tests are designed to reflect the direct toxic effects of a chemical substance on the test organism, for example the immobilization of Daphnia (OECD method 202) or the inhibition of algal growth (OECD method 201). In algal tests specifically, ENPs may indirectly inhibit growth by changing the pH, redox conditions, nutrient or light availability, or cause direct physical effects such as disruption of cell walls and membranes as a result of their topography and surface properties (Hartmann et al. 2013).

For platinum nanoparticles (PtNPs), which create very dark suspensions, algal growth rate inhibition has been observed in the standard ISO 8692 algal test with *P. subcapitata*. When separating algae from the PtNP suspensions through a double-vial system (Figure 1, I) concentration-dependent growth rate inhibition was still observed (Sørensen et al. 2014). However, the obtained EC_{50} value was higher when tested in the double-vials compared to the standard setup (Figure 1, H) indicating that PtNPs inhibit algal growth rates by other means than shading. Conversely, a test with physical separation of a TiO_2 ENP suspension and algae did not indicate shading effects and could therefore not explain the observed growth inhibition caused by these particles under standard conditions (Hartmann et al. 2010). Nonetheless, such tests cannot account for algae cell encapsulation by ENPs which can occur upon direct contact. This shading on a cellular level may therefore be a potential effect mechanism (Hartmann et al. 2010). Consequently, growth rate inhibition alone may not be an appropriate endpoint for testing ENP toxicity, as it is not possible to clearly discriminate between direct toxic effects and indirect physical effects.

The adhesion of ENPs to the surface of cells and organisms may also pose an issue in other test organisms than algae, for example crustaceans and fish. The ENPs may adhere to the exoskeleton and the antenna of *D. magna* or to the gills of fish, thereby causing effects to the organisms, as a result of physical interactions. As an example, *D. magna* exposed to PtNPs in the OECD immobilization test resulted in substantial adhesion of PtNPs to the exoskeleton of the organisms

apparently affecting their mobility. To differentiate between immobility and lethality, both endpoints were recorded, resulting in lower effective concentrations for immobilization (Sørensen et al., 2014). As immobility may be affected by physical effects, lethality appears to be a more appropriate endpoint. However, the lethality observed in this test may also be influenced by the immobility experienced by the organisms. Daphnids are pelagic filter feeders but will also search for food along bottom sediments. Under laboratory conditions a similar behavior is observed, resulting in increased contact with sedimented ENPs. In an attempt to overcome this confounding factor, exposure beakers with inserted mesh-bottomed testing chambers were used. This allowed for exposure of daphnids to the PtNP suspension, but hindered contact with larger aggregates and sedimented PtNPs (Figure 1, J-K). This setup resulted in markedly less PtNP adhesion to the exoskeletons of the daphnids and similar IC_{50} and LC_{50} values. The outcome of this test setup reflected PtNP toxicity rather than the physical interaction caused by agglomeration and sedimentation under the test conditions.

In addition to interference with the test endpoints itself, the presence of ENPs may also interfere with the analytical methods used in standard testing. This is the case for methods used for quantification of algal growth (Hartmann et al., 2013) as well as the seemingly simple visual evaluation of *D. magna* immobility which often can be difficult in the presence of darkly colored ENPs obscuring the suspension.

Overall, the difference in physical and chemical behavior of suspended ENPs compared to dissolved compounds needs to be considered when conducting aquatic toxicity testing of ENPs. It is crucial to carefully evaluate the test outcome and the appropriateness of applied analytical methods, exposure period, media, test setups, and endpoints. The toxicity tests mentioned above exemplify the occurrence of both physical and chemical effects in standard ecotoxicity tests. Modifications to the test setups may assist to explain the underlying mechanisms for the observed responses and help distinguish physical from chemical toxicological effects. Problems may arise when results from ENP ecotoxicity tests using current standardized methods are used as intended, namely for the identification and comparison of the toxicity of different compounds, without considering the limitations of these data. Furthermore, physical interferences may be considered a testing artefact stemming from the use of high concentrations and the data obtained will have very limited value for extrapolation purposes such as determination of PNEC values (Baun et al. 2009).

How we choose to deal with physical interference from ENPs in standardized ecotoxicity tests is another issue. Physical effects must be considered as a confounding factor rather than an actual effect under the current standard testing scheme. A shift in test paradigm may therefore be necessary to embrace the fact that physical effects of ENPs can contribute to the observed biological effects in other ways than what is known for dissolved chemicals. For both scientific and regulatory purposes it is important to have a good understanding of test outcomes and potential limitations of data. We recommend that physical effects of ENPs from standard testing are investigated and accounted for and attempts should be made to minimize the amount of confounding factors by stabilizing ENP suspensions for testing, e.g. by consideration of factors such as media, pH, natural organic matter, aging, exposure timing, and endpoints (Sørensen and Baun 2015; Cupi et al. 2015).

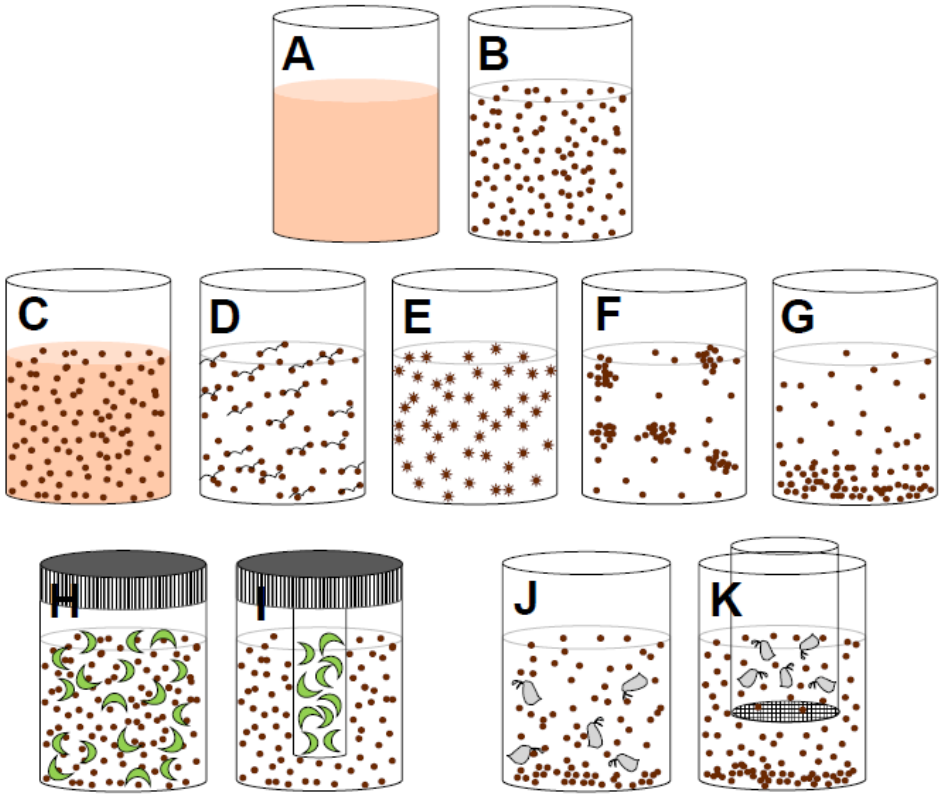

Figure 1. Illustrations of the inherent difference between a chemical solution and an ENP suspension (A and B), the ENP specific processes likely to occur during aquatic toxicity tests (C-G) and test setups for distinguishing physical and chemical effects (H-K). Test guidelines are developed for dissolving chemicals, remaining stable during incubation (A), whereas ENPs are suspended rather than dissolved (B) and change over time due to dissolution (C), interactions with medium components and organism exudates (D), transformations of surface coatings (E), aggregation/agglomeration (F), and sedimentation (G). In regular test setups for algae (H) and daphnia (J), the occurrence of such processes (C-G) may interfere with the test outcome and cause physical effects. Physical shading effects in algal tests may be investigated by a double-vial setup (I), where algae are contained in the smaller inner-vial, surrounded by the ENP suspension in larger outer-vial. Physical immobilization of daphnids arising from contact with larger aggregated/sedimented ENPs can be avoided by keeping the daphnids in a mesh-bottomed beaker inserted into larger beakers containing ENP suspensions (K).

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